Exhibit 7

Role of p38 Mitogen-Activated Protein Kinase in a Murine Model of Pulmonary Inflammation[1]

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Early inflammatory events include cytokine release, activation, and rapid accumulation of neutrophils, with subsequent recruitment of mononuclear cells. The p38 mitogen-activated protein kinase (MAPK) intracellular signaling pathway plays a central role in regulating a wide range of inflammatory responses in many different cells. A murine model of mild LPS-induced lung inflammation was developed to investigate the role of the p38 MAPK pathway in the initiation of pulmonary inflammation. A novel p38 MAPK inhibitor, M39, was used to determine the functional consequences of p38 MAPK activation. In vitro exposure to M39 inhibited p38 MAPK activity in LPS-stimulated murine and human neutrophils and macrophages, blocked TNF-α and macrophage inflammatory protein-2 (MIP-2) release, and eliminated migration of murine neutrophils toward the chemokines MIP-2 and KC. In contrast, alveolar macrophages required a 1000-fold greater concentration of M39 to block release of TNF-α and MIP-2. Systemic inhibition of p38 MAPK resulted in significant decreases in the release of TNF-α and neutrophil accumulation in the airspaces following intratracheal administration of LPS. Recovery of MIP-2 and KC from the airspaces was not affected by inhibition of p38 MAPK, and accumulation of mononuclear cells was not significantly reduced. When KC was instilled as a proinflammatory stimulus, neutrophil accumulation was significantly decreased by p38 MAPK inhibition independent of TNF-α or LPS. Together, these results demonstrate a much greater dependence on the p38 MAPK cascade in the neutrophil when compared with other leukocytes, and suggest a means of selectively studying and potentially modulating early inflammation in the lung. *The Journal of Immunology*, 2000, 164: 2151–2159.

The rapid accumulation of neutrophils to the lung in response to a proinflammatory stimulus is one of the first recognizable events in the pathogenesis of many pulmonary diseases. The process by which neutrophils cross the pulmonary vasculature, migrate through the lung interstitium, and ultimately accumulate in the airways requires complex interactions between circulating leukocytes and the cells of the lung (1). Although many aspects of neutrophil accumulation are poorly understood, a number of discrete events have been identified. In health, a significant proportion of the circulating neutrophils are passing through the lung capillary bed at any point in time, contributing to the marginating pool (2). In the setting of lung injury, effective migration and accumulation of neutrophils into the airspaces requires coordinated responses including up-regulation of adhesion molecules, cytoskeletal rearrangement, increases in cell size and stiffness, and chemotaxis (2–4). To a large extent, cytokines and other soluble proinflammatory stimuli orchestrate the responses of the leukocytes. Synthesis of cytokines by the neutrophil itself may serve to amplify and perpetuate the recruitment of leukocytes to the airspaces in certain disease states (5). Monocyte and macrophage accumulation in the lung typically occurs following an initial recruitment of neutrophils. In some animal models of acute inflammation, accumulation of monocytes in the lung was found to be neutrophil dependent (6).

Of particular interest is the ability of LPS to induce lung inflammation, as local or systemic endotoxin release is an important feature of many diseases, including focal pneumonias, cystic fibrosis, and the acute respiratory distress syndrome. LPS is not an effective chemoattractant for neutrophils, but can trigger an inflammatory cascade via the synthesis of cytokines and other proinflammatory mediators by resident alveolar macrophages (AM),[3] local mast cells, fibroblasts, epithelia, and endothelial cells. The release of TNF-α and neutrophil-directed chemokines such as IL-8 are essential to early LPS-mediated neutrophil recruitment.

The combined effects of TNF-α and IL-8 on neutrophil recruitment are complex and incompletely understood. Known roles of TNF-α include activation of endothelial cells to express adherence proteins, induction of an array of secondary inflammatory mediators, and "priming" of neutrophils for enhanced phagocytic and bactericidal activity (7). Through studies with specific Abs and genetically modified mice, the requirement for TNF-α in the pathogenesis of LPS-induced shock and tissue injury has been confirmed. However, these techniques do not allow for selective reduction of the release of TNF-α by a cell type, nor modulation of the ability of neutrophils to respond to TNF-α. As a single agent, TNF-α also does not induce chemotaxis of neutrophils. However, the ELR(+)CXC chemokine IL-8 is one of the most specific neutrophil chemoattractants yet described (8–10). IL-8 has not been

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[3] Abbreviations used in this paper: AM, alveolar macrophages; ATF-2, activated transcription factor-2; BAL, bronchial alveolar lavage; ERK, extracellular signal-regulated kinases; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MIP-2, macrophage inflammatory protein-2; MPO, myeloperoxidase.

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identified in mice. but macrophage inflammatory protein-2 (MIP-2) and KC share the same ELR(+)CXC structure and act as functional homologue of human IL-8 (11, 12).

Selective responses of cells to external stimuli may be understood through differential activation of intracellular signaling mechanisms. The mitogen-activated protein kinase (MAPK) superfamily are highly conserved signaling kinases that regulate cell growth, differentiation, and stress responses (13). At least three distinct families of MAPKs exist in mammalian cells: the p42/44 extracellular signal-regulated kinase (ERK) MAPKs, c-Jun NH_2-terminal kinases (JNKs), and p38 MAPK (14–16). Both the coordinated release of cytokines by host defense cells and the functional response of neutrophils to cytokines and other proinflammatory agents are to varying degrees regulated by p38 MAPK. In the neutrophil, p38α MAPK is activated in response to many stimuli, including LPS and TNF-α (17, 18). Once activated, p38α MAPK is capable of modulating functional responses through phosphorylation of transcription factors and activation of other kinases. In LPS-stimulated neutrophils, p38α MAPK regulates distinctly different functions, including adhesion, activation of NF-κB, and the synthesis of TNF-α and IL-8 (19–21). In neutrophils stimulated with the chemoattractant FMLP, activation of p38α MAPK regulates both superoxide anion release and chemotaxis (19, 20). In the LPS-stimulated monocyte/macrophage, inhibition of p38α MAPK blocks TNF-α and IL-8 release (16, 22). In cells other than leukocytes, p38 MAPK also regulates stress-responses, including release of IL-8 by bronchial epithelial cells in response to TNF-α or other inflammatory stimuli (23). LPS stimulation also causes activation of p38 MAPK in endothelial cells, resulting in up-regulation of the ICAM-1 adhesion molecule (24).

Given this central role of p38 MAPK as a regulator of multiple inflammatory responses in many diverse cell types, we questioned the effect of in vivo p38 MAPK inhibition on neutrophil accumulation in the lung. For these studies, we employed a murine model of mild pulmonary inflammation in response to a single administration of LPS in the airspace. Inhibition of p38α MAPK was accomplished with the novel compound M39, which is the most highly selective and potent inhibitor of p38 MAPK described to date (25). We studied the effects of selective inhibition of p38 MAPK on several events critical in the pathogenesis of the early inflammatory response of the murine lung. Herein we report that in vitro inhibition of p38 MAPK resulted in a significant decrease in murine neutrophil function, but a limited effect on other inflammatory responses. In vivo, this resulted in the loss of initial neutrophil recruitment to the airspaces, while later accumulation of mononuclear cells remained largely intact. Together, these data indicate the potential for relatively selective in vivo inhibition of neutrophilic responses.

Materials and Methods

Materials

Endotoxin-free reagents and plastics were used in all experiments. Aprotinin, leupeptin, Tris-HCl, Triton X-100, igepal, PMSF, EDTA, EGTA, Nonidet P-40, and protein A-Sepharose were purchased from Sigma (St. Louis, MO), and [γ-^{32}P]ATP was purchased from Amersham (Arlington Heights, IL). M39 [(*S*)-5-[2-(1-phenylethylamino)pyrimidin-4-yl]-1-methyl-4-(3-trifluoromethylphenyl)-2-(4-piperidinyl) imidazole] was provided by Merck (Rahway, NJ) and stored in DMSO at −20°C. LPS was purified from *Escherichia coli* 0111:B4 (List Biological Laboratories, Campbell, CA). Recombinant KC and MIP-2 were purchased from R&D Systems (Minneapolis, MN). Activated transcription factor (ATF)-2_{1-110} was prepared as previously described (17, 19).

Animals

Female C57BL/6 mice (Harlan Sprague Dawley, Indianapolis, IN), 6–12 wk of age and weighing 16–20 g, were used in all experiments. They were given commercial pellet food and water ad libitum. All experiments were performed in accordance with the Animal Welfare Act and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals after review of the protocol by the Animal Care and Use Committee of the National Jewish Medical and Research Center. Anesthesia was provided by a single i.p. injection of 333 mg/kg avertin. Avertin was prepared by mixing 10 g tribromoethyl alcohol (Aldrich, Milwaukee, WI) with 10 ml teriary amyl alcohol (Aldrich) and diluting this stock to a 2.5% solution in sterile saline.

Murine bronchial alveolar lavage (BAL)

BAL was performed immediately following sacrifice of the animals by pentobarbital overdose or cervical dislocation. The procedure was performed with the lungs in situ but with the chest cavity opened by midline incision. The trachea was intubated orally or directly through a small cut-down of the skin overlying the trachea with a 20-g angiocath (Baxter Quik-Cath, Baxter Health Care, Deerfield, IL). Two to four 0.8-ml aliquots of saline with 20 U/ml heparin were instilled and sequentially removed by gentle hand suction with a 1-ml syringe. The volume of BAL recovered was quantified and cells recovered were counted in a hemocytometer. Cell types were determined by Wright staining of a spun sample. All slides were counted twice by different observers blinded to the status of the animal. Samples for cytokine analysis were immediately frozen in a dry ice/ethanol bath and stored at −70°C.

Isolation of cells

Human neutrophils were isolated by the plasma Percoll method (26) and suspended in Krebs-Ringer phosphate buffer with 0.2% dextrose at pH 7.2 or in RPMI 1640 culture medium (BioWhittaker, Walkersville, MD). Mature murine bone marrow neutrophils were isolated from mouse femurs and tibias. Animals were sacrificed by cervical dislocation, and the bones were dissected. Both ends of each bone were removed, and a 25-gauge needle on a 5-ml syringe containing HBSS (without calcium, magnesium, bicarbonate, or phenol red) was employed to express marrow from the bones. Marrow cords were collected in a 50-ml polypropylene conical tube (Becton Dickinson, Bedford, MA) and subsequently resuspended by gentle aspiration of the suspension through a 19-gauge needle. The marrow cells were pelleted by centrifugation at 112 × *g* for 6 min, washed once with HBSS, and resuspended in HBSS to a final volume of 2 ml in preparation for density gradient centrifugation. A stock solution of Percoll (100% fine grade; Pharmacia Fine Chemicals, Piscataway, NJ) was prepared in 10× HBSS in a ratio of 9:1 (v/v) Percoll:10× HBSS. A 3 × 2-ml Percoll discontinuous density gradient (72, 64, and 52% with 1× HBSS) was prepared in a 15-ml polypropylene conical tube (Becton Dickinson). The marrow suspension was layered on top of the Percoll gradient and centrifuged at 1060 × *g* for 30 min. Morphologically mature appearing neutrophils at a concentration of >95% formed a band at the interface of the 64 and 72% Percoll layers. This band was carefully aspirated and mixed with 12 ml of 1× HBSS in a 15-ml conical tube, centrifuged at 112 × *g* for 6 min, washed twice with 1× HBSS, and resuspended in 1× HBSS to a volume of 2 ml and counted by hemocytometer. Typical yields were ~1–2 × 10^7 mature bone marrow neutrophils per mouse. In separate studies, the marrow neutrophils were shown to have equivalent functional responses and recirculation patterns when compared with peripheral murine neutrophils (B.T.S., unpublished observations). Murine peripheral blood neutrophils were isolated by modification of methods previously reported (27) for the purification of rabbit peripheral neutrophils. Mice were volume expanded and exsanguinated into a 3.8% citrate solution followed by centrifugation at 300 × *g* for 20 min. The cell pellet was resuspended in 6% dextran and 0.9% NaCl solution (in a ratio of 1:5.25, dextran:saline) to a final volume of 150% the original blood volume and sedimented at unity gravity for 30 min. The leukocyte-rich supernatant was aspirated, washed once in HBSS, layered over a Percoll gradient (78, 66, and 54%) and centrifuged at 1060 × *g* for 30 min. Cytospun samples of the dense band revealed >90% neutrophils. Following lysis with hypotonic saline, typical yields were ~2–4 × 10^5 peripheral blood neutrophils per mouse. Trypan blue dye exclusion showed the cells to be >97% viable following purification. Murine AM were isolated by two sequential BALs. Typical yields were ~2 × 10^5 cells per mouse and were 97–99% AM as assessed by Wright staining of spun samples.

Neutrophil functional assays

All experiments were done in the presence of 1% human or murine heat-inactivated platelet-poor plasma. Cytokine release assays were performed with murine neutrophils isolated from peripheral blood or murine AM resuspended in RPMI 1640 containing 2% murine heat-inactivated platelet-poor plasma at a concentration of 5 × 10^6 cells/ml. One milliliter of cells

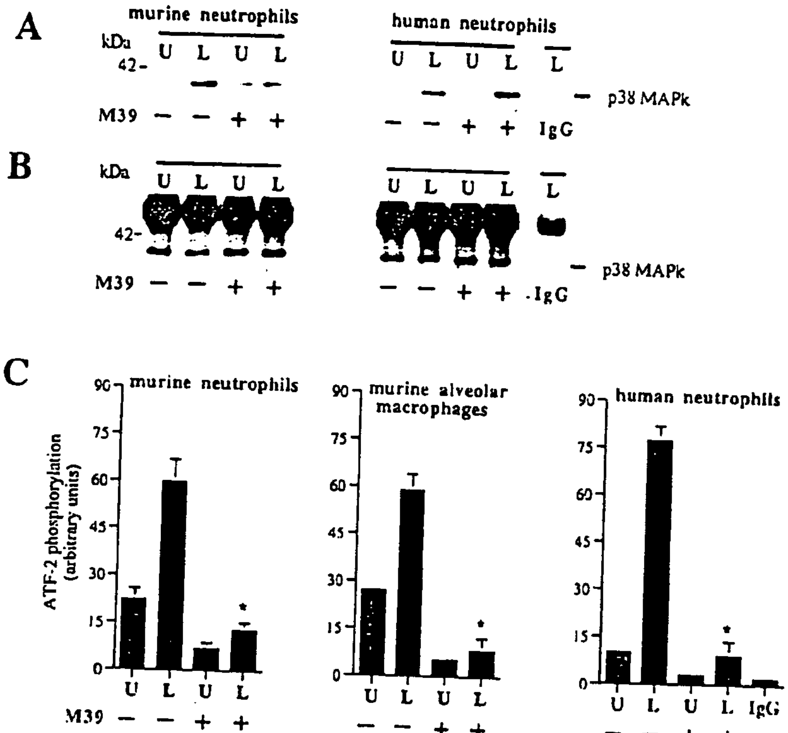

FIGURE 1. M39 inhibits LPS-induced activation of p38α MAPK in the murine neutrophil. *A*, Tyrosine phosphorylation of p38α MAPK. Murine and human neutrophils (4×10^6 per condition) were exposed to M39 (1 μM) for 60 min at 37°C or left untreated, then stimulated with LPS (100 ng/ml) for 20 min at 37°C (L) or left unstimulated (U). The cells were lysed, and p38α MAPK was immunoprecipitated and separated by SDS-PAGE. Western blots were probed with an anti-phosphotyrosine Ab capable of reacting with phosphorylated tyrosine residues of p38α MAPK. A nonimmune IgG control is shown in *lane 5* for human neutrophils to confirm the specificity of our p38α MAPK antiserum. *B*, Equivalent immunoprecipitation of p38α MAPK. Blots from *A* were reprobed with an anti-p38 MAPK Ab to demonstrate an equal amount of p38 MAPK immunoprecipitated for each condition studied. *C*, Activation of p38α MAPK. p38α MAPK was immunoprecipitated from the lysates depicted in *A* and *B* and combined with ATF-2_{1-110} in the presence of [^{32}P]ATP. The peptide was subjected to SDS-PAGE, and the amount of [^{32}P] phosphorylation of ATF-2_{1-110} was assessed by phosphor screen autoradiography. Under identical conditions, p38α MAPK immunoprecipitated from murine AM was also analyzed for inhibition by M39. Immunoprecipitation with the nonimmune IgG control shown in *lane 5* of *A* and *B* for human neutrophils confirmed a lack of nonspecific phosphorylation of ATF-2_{1-110}. Plots depict means ± SEM from three consecutive experiments expressed in arbitrary units. *, $p < 0.01$ by Student's *t* test, compared with the LPS-stimulated sample in the absence of M39 for each cell type.

suspension was added per well of a 12-well flat-bottom tissue culture-treated polystyrene plate (Costar, Corning, NY). Cells were allowed to settle without agitation for 60 min at 37°C (in the presence or absence of the p38 MAPK inhibitor), followed by addition of stimuli for the designated periods. At the end of the stimulation, the supernatant was removed for quantification of KC, MIP-2, or TNF-α by immunoassay (R&D Systems). In vitro inhibition of p38 MAPK was performed by incubation of neutrophils or macrophages with M39 over a range of concentrations for 60 min at 37°C. Collagen gel migration assays were performed as previously described (27) with minor modifications. Throughout all migration assays, Krebs-Ringer-phosphate dextrose with 0.25% human serum albumin was used as the buffer. Between 2 and 5×10^6 neutrophils were loaded per gel for each condition studied. Gels were inverted onto mounting media containing 0.1% *p*-phenylenediamine and 70% glycerol with 1 drop of defined diameter fluorescent beads (DNA-Check; Coulter, Hialeati, FL) to establish the scale. All gels were examined with a ×40 dry objective and numerical aperture 0.55. Diagrams of cells in each gel section were made at 5-μm intervals, and the number of cells at each depth were recorded. A minimum of three gel sections were examined for each condition, and averages were calculated for each depth. Values for cell distribution at each depth were expressed as a percent of total cells observed in the entire vertical section.

P38 MAPK immunoprecipitation assays

Kinase activity of p38α MAPK was assayed from immunoprecipitated samples by the ability to phosphorylate ATF-2_{1-110} as previously described (19).

Intratracheal instillation of proinflammatory stimuli

Following anesthesia with avertin, a 300-ng aliquot of LPS or 1 μg KC dissolved in 50 μl saline containing 0.1% human serum albumin was injected into the mouse airways by passing a 22-gauge bent feeding needle with a 1.25-mm ball diameter (Popper & Sons, New Hyde Park, NY) through the oropharynx into the trachea.

In vivo inhibition of p38 MAPK

Anesthetized mice were administered M39 by gastric intubation of a 22-gauge straight feeding needle with a 2.25-mm ball diameter (Popper & Sons). Fasting mice were placed in a semiupright position, and M39 suspended in 100 μl hydroxypropylmethylcellulose (Abbott Laboratories, Abbott Park, IL) was instilled at a dose of 3 mg/kg. The M39 was administered 2 h before and 12 h following intratracheal instillation of KC or LPS, except for time points earlier than 12 h, in which a single dose of M39 was administered.

Histological examination and quantification of neutrophil accumulation of murine lung tissue

Animals were treated with saline or LPS in the presence or absence of M39. At 24 h, the animals were sacrificed by pentobarbital overdose and a midline incision was performed. A 20-g catheter (Baxter Health Care) was inserted into the trachea and secured by tying with 2–0 silk followed by careful dissection to remove the lungs from the thoracic cavity. After full inflation with air to 25 cm water pressure, the trachea was tied and the lungs submerged in 1.5% glutaraldehyde solution in sodium cacodylate buffer for 24 h. Sections (4 μm) taken across the entire lung were embedded in paraffin. Sagittal sections were stained with hematoxylin-eosin and examined by light microscopy. Between two and four animals were studied for each condition, and representative sections of lungs were chosen by two

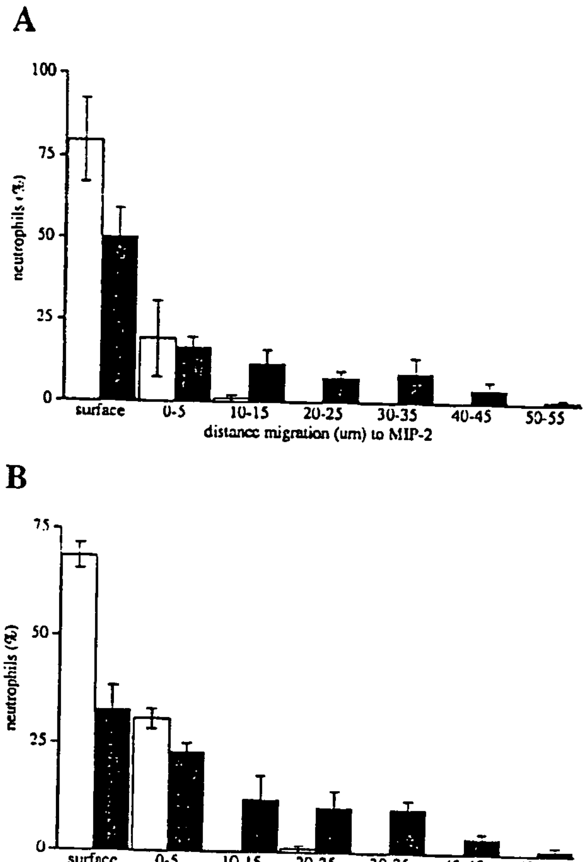

FIGURE 2. Inhibition of p38 MAPK blocks neutrophil migration toward MIP-2 and KC. *A*, Neutrophils (3×10^6) were treated with 1 μM M39 (□) or left untreated (■) for 60 min at 37°C and then subjected to an in vitro collagen gel migration assay (see *Materials and Methods*) toward MIP-2 (10^{-7} M) for 75 min at 37°C. Each plot depicts distance of migration in 10-μm intervals, expressed in mean percent of total cells counted ± SEM from three consecutive experiments, with three independent readings at each depth. Cells that failed to penetrate the gel were quantified in the interval labeled "surface." The failure of neutrophils to migrate beyond the gel surface following inhibition of p38 MAPK was significant ($p < 0.0001$) by χ^2 analysis when compared with untreated cells. *B*, Under identical conditions, neutrophils were treated with M39 (□) or left untreated (■) and then subjected to an in vitro collagen gel migration assay toward KC (10^{-7} M). The observed loss of migration following inhibition of p38 MAPK was significant ($p < 0.0001$) by χ^2 analysis.

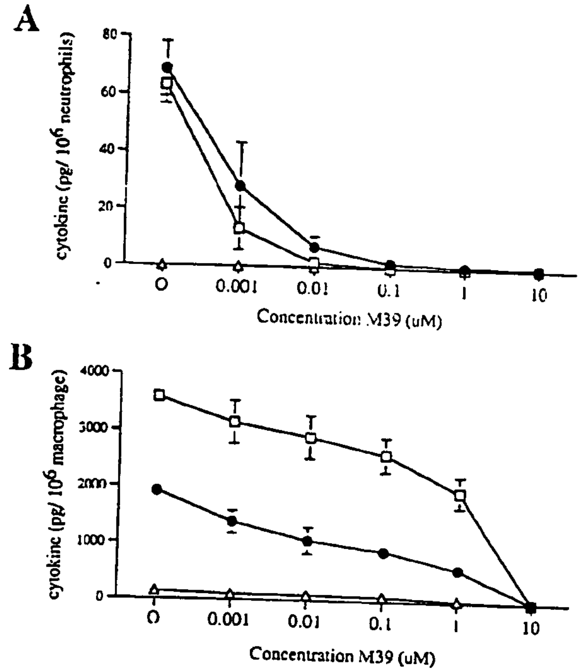

FIGURE 3. Effect of p38 MAPK inhibition on cytokine release of LPS-stimulated murine neutrophils and AM. *A*, Murine neutrophils were treated with M39 (0.001–10 μM at 37°C) or left untreated and allowed to adhere to plastic. The cells were then stimulated with LPS (100 ng/ml) for 4 h at 37°C, and the quantity of TNF-α (●), MIP-2 (□), or KC (△) released was quantified. Each plot depicts mean cytokine release (pg per 10^6 cells) ± SEM for each concentration of M39 from three consecutive experiments. The M39 concentration-dependent inhibition of cytokine release is significant for TNF-α and MIP-2 ($p < 0.001$) by one-way ANOVA, but not for KC. *B*, Under identical conditions, murine AM were treated with M39 or left untreated and allowed to adhere to plastic. The cells were then stimulated with LPS (100 ng/ml) for 4 h at 37°C and the quantity of TNF-α (●), MIP-2 (□), or KC (△) released was quantified. Each plot depict mean cytokine release (pg per 10^6 cells) ± SEM for each concentration of M39 from three consecutive experiments. The M39 concentration-dependent inhibition of cytokine release is significant for TNF-α and MIP-2 ($p < 0.001$) by one-way ANOVA, but not for KC.

independent observers blinded to the treatment status of the animals. Photomicrographs were taken at ×400 magnification. Quantification of neutrophil accumulation in the whole lung excluding the airspaces was performed by myeloperoxidase (MPO) assay as previously described (28) with minor modifications. Following BAL, isolated whole lungs were frozen in liquid nitrogen, weighed, and then homogenized. Following centrifugation at 20,000 × *g* for 30 min, the insoluble pellet was resuspended in 50 mM potassium phosphate buffer, pH 6.0, with 0.5% hexadecyltrimethylammonium bromide. Samples were sonicated, incubated at 60°C for 2 h, and assayed for activity in a hydrogen peroxide/*o*-dianisidine buffer at 460 nm. Results are expressed as units of MPO activity per gram of lung tissue.

Statistical analysis

Data were analyzed using JMP statistical software (SAS Institute, Cary, NC). Student's unpaired *t* test (two-tailed) was use to determine significance of p38 MAPK inhibition (Fig. 1) and neutrophil accumulation and MPO content (Fig. 8) for a single time point. Differences in chemotaxis (Fig. 2) were analyzed by a χ^2 test. One-way ANOVA was used to analyze the effect of LPS-induced cytokine release and leukocyte accumulation over time (Figs. 3 and 4). Differences in in vivo cell accumulation and cytokine release over time in the presence and absence of p38 MAPK inhibition (Figs. 5, 6, and 9) were analyzed by two-way ANOVA. When a significant interaction between inhibition and time existed, the effect of inhibition was analyzed separately for each time point. For all tests, $p < 0.01$ was considered significant unless otherwise indicated.

Results

Inhibition of p38α MAPK activation in murine neutrophils

Previous reports have demonstrated phosphorylation and activation of p38α MAPK in human neutrophils following stimulation with LPS (17, 18). To determine whether activation of p38α MAPK occurs in murine neutrophils in response to LPS, and assess the ability of M39 to inhibit p38α MAPK activity, murine bone marrow neutrophils were stimulated with LPS in the presence and absence of M39. Activity and phosphorylation of p38α MAPK were assessed simultaneously by immunoprecipitation of the kinase from neutrophil lysates stimulated with LPS or left unstimulated. The p38α MAPK was resolved by SDS-PAGE, and the Western blot was stained with an Ab capable of detecting tyrosine phosphorylation of p38α MAPK (Fig. 1*A*). For comparison, an equal number of human neutrophils were stimulated and analyzed

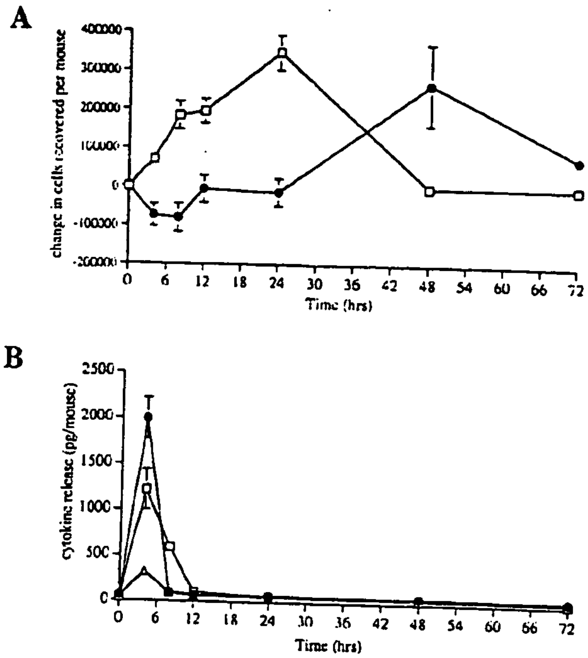

FIGURE 4. Features of pulmonary inflammation induced by intratracheal administration of LPS. *A*, Influx of leukocytes in the murine airspaces in response to intratracheal LPS. Mice were administered LPS (300 ng) at time 0, and the difference in numbers of neutrophils (□) and mononuclear cells (●) recovered by BAL compared with baseline levels was plotted over a series of time points. Each data point represents the mean change in number of cells ± SEM from three to five animals. The effect of LPS on leukocyte accumulation over time is significant by one-way ANOVA for both neutrophils ($p < 0.001$) and monocytes ($p = 0.003$). *B*, Cytokines in the murine airspaces in response to LPS. BAL samples recovered following administration of LPS (300 ng) were analyzed for TNF-α (●), MIP-2 (□), and KC (△). Quantities of cytokine recovered per mouse were plotted against time (see *Materials and Methods*). Each data point represents the mean amount ± SEM of cytokine recovered from the BAL of three to five animals. The effect of LPS on cytokine release over time is significant by one-way ANOVA ($p < 0.001$).

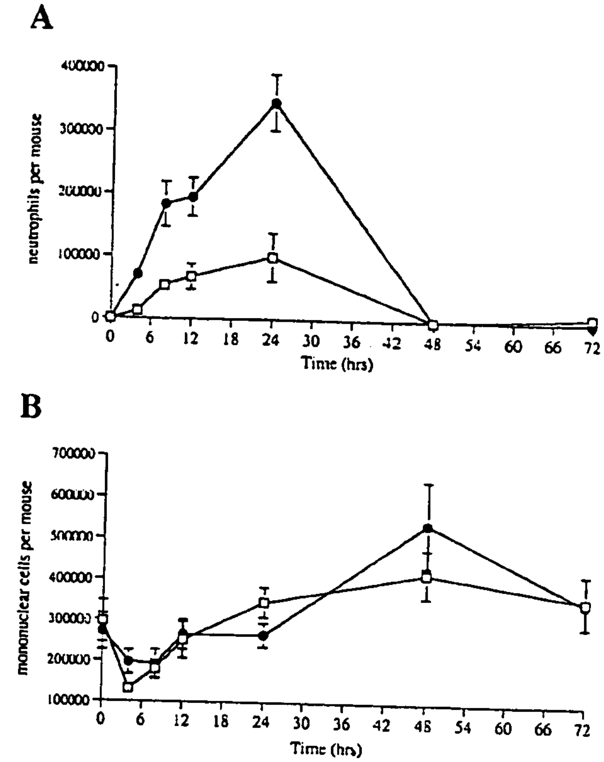

FIGURE 5. Effect of in vivo inhibition of p38 MAPK on leukocyte accumulation in the airspaces. *A*, Neutrophil accumulation in the murine airspaces following intratracheal LPS. Mice pretreated with M39 (□) were administered LPS (300 ng) at time 0, and cell analysis of BAL was compared with untreated mice (●) over a series of times (see *Materials and Methods*). Each point represents mean number of neutrophils ± SEM from three to five animals. The effect of M39 on LPS-induced neutrophil accumulation over time is significant ($p = 0.0005$) by two-way ANOVA. *B*, Mononuclear cells accumulation in the murine airspaces following LPS. Mononuclear cells from BAL samples depicted in Fig. 4*A* were analyzed in mice administered M39 (□) and compared with untreated mice (●) over a series of times. Each point represents mean number of cells ± SEM from three to five animals. The effect of M39 on LPS-induced mononuclear cell accumulation over time is not significant ($p = 0.39$) by two-way ANOVA.

in the identical manner (Fig. 1*A*). The blot was then reprobed with a second Ab against p38α MAPK, confirming that equivalent amounts of kinase were immunoprecipitated for each condition (data not shown). Activity of p38α MAPK was determined by combining immunoprecipitated p38α MAPK with ATF-2_{1-110}, a known substrate (29), in the presence of [^{32}P]ATP (Fig. 1*B*). LPS stimulation resulted in robust tyrosine phosphorylation of p38α MAPK in both murine and human neutrophils. However, p38α MAPK isolated from LPS-stimulated cells treated with M39 had significantly reduced kinase activity. Inhibition of p38 MAPK by M39 may result in varying degrees of decreased tyrosine phosphorylation between different cell types and species (our unpublished observations). These results demonstrate phosphorylation and activation of p38α MAPK in the murine neutrophil and the ability of M39 to inhibit LPS-induced activation of p38α MAPK.

Inhibition of p38 MAPK blocks chemokine-induced chemotaxis of murine neutrophils

Chemotaxis is a complex response involving coordination of adhesion and actin assembly. We have reported previously that inhibition of p38 MAPK results in loss of chemotaxis response by human neutrophils to FMLP (19). We tested the effect of p38 MAPK inhibition on migration of murine neutrophils toward the chemoattractants MIP-2 and KC. Neutrophil chemotaxis through a three-dimensional collagen matrix was quantified by counting the number of cells within a series of 5-μm intervals after 75 min of exposure to the chemokines. In the presence of M39, neutrophil chemotaxis to MIP-2 (Fig. 2*A*) and KC (Fig. 2*B*) was blocked.

Effect of p38 MAPK inhibition on cytokine release of LPS-stimulated murine neutrophils and AM

An important role of AM is cytokines release in response to LPS, thus triggering and coordinating early inflammation. Neutrophils also have the capability to synthesize and release a limited number of cytokines (30) and under certain conditions may be important in perpetuating the inflammatory response. Activation of p38 MAPK has been associated with cytokine production by both monocytes/macrophages and neutrophils. We tested the effect of p38 MAPK inhibition with M39 on LPS-induced release of TNF-α, MIP-2, and KC from adherent neutrophils and AM (see *Materials and Methods*). An IC_{50} of M39 inhibition of TNF-α and MIP-2 release by LPS-stimulated neutrophils was achieved with a concentration of M39 <0.1 nM (Fig. 3*A*). In contrast, the IC_{50} of M39 for LPS-activated AM to achieve inhibition of TNF-α and MIP-2 release

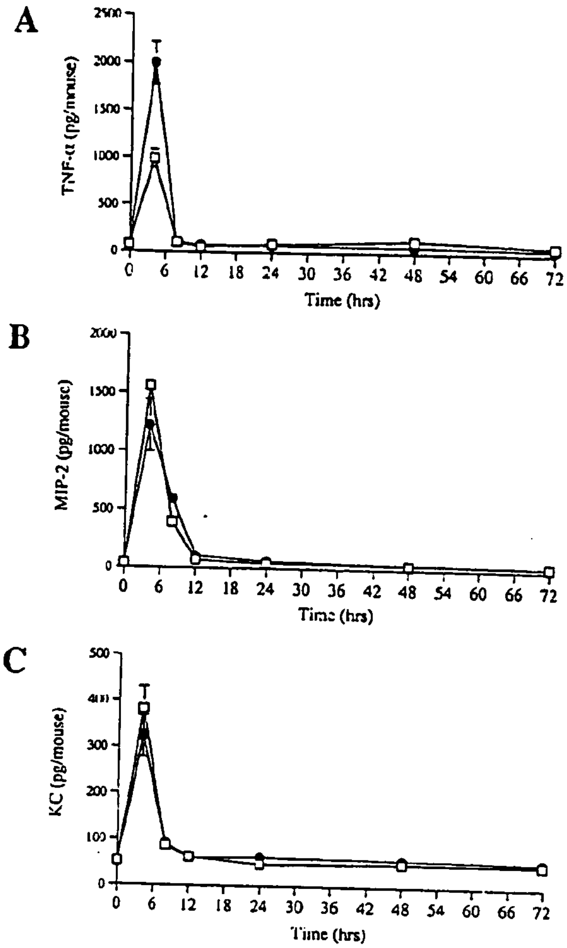

FIGURE 6. Effect of in vivo inhibition of p38 MAPK on cytokine release in the airspaces. *A*, TNF-α in the murine airspaces in response to LPS. Untreated mice (●) were administered LPS (300 ng) at time 0, and the quantity of TNF-α in the BAL were compared with mice pretreated with M39 (□) over a series of times (see *Materials and Methods*). Each point represents the mean amount (pg/mouse) ± SEM of TNF-α recovered from the BAL from three to five animals. *, $p < 0.01$, compared with M39 treated mice at the same time point. The effect of M39 on LPS-induced TNF-α release over time is significant ($p < 0.0001$) by two-way ANOVA. *B*, MIP-2 in the murine airspaces in response to LPS. BAL samples from Fig. 5*A* were reanalyzed for MIP-2 in untreated mice (●) compared with mice administered M39 (□) over a series of times. Each point represents the mean amount (pg/mouse) ± SEM of MIP-2 recovered from the BAL from three to five animals. The effect of M39 on LPS-induced MIP-2 release over time is not significant ($p = 0.49$) by two-way ANOVA. *C*, KC in the murine airspaces in response to LPS. BAL samples from Fig. 5*A* were reanalyzed for KC in untreated mice (●) compared with mice administered M39 (□) over a series of times. Each point represents the mean amount (pg/mouse) ± SEM of KC recovered from the BAL from three to five animals. The effect of M39 on LPS-induced KC release over time is not significant ($p = 0.73$) by two-way ANOVA.

was >1000-fold higher (Fig. 3*B*). Neither LPS-stimulated neutrophils nor AM were found to release significant quantities of KC under the conditions studied (Fig. 3, *A* and *B*). Viability of neutrophils and macrophages treated with 10 μM M39 ranged from 97 to 99%, equal to the viability of the untreated cells (data not shown). These results support the conclusion that in vitro inhibition of p38 MAPK may result in a relatively greater loss of functional response by the neutrophil than by the AM.

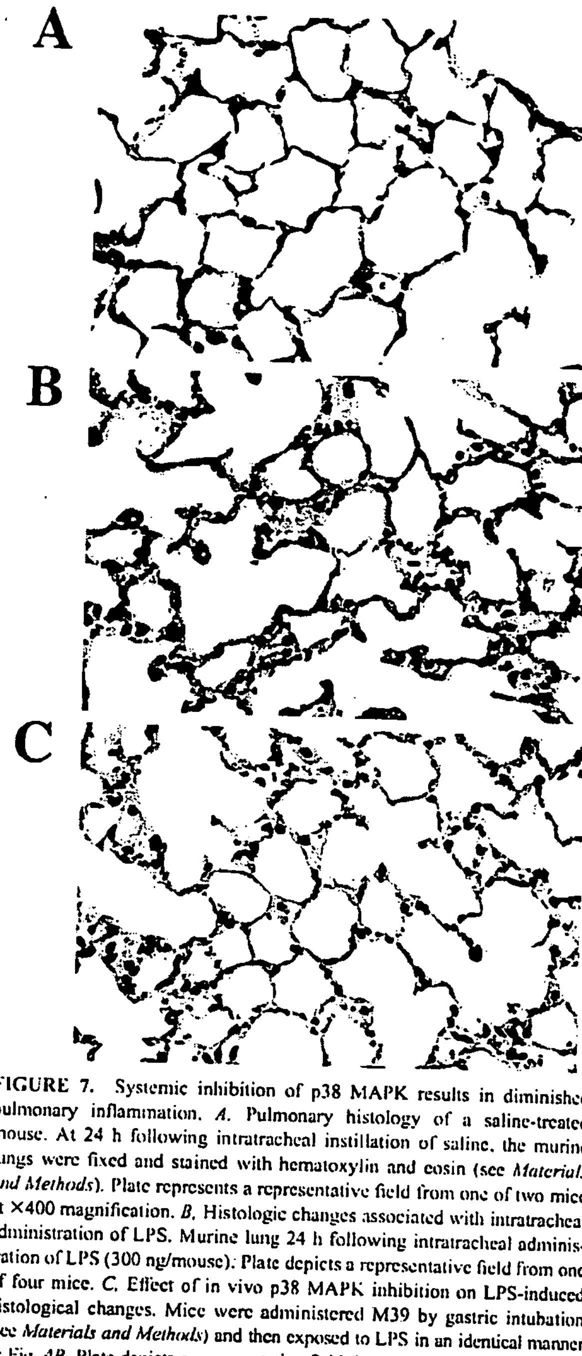

FIGURE 7. Systemic inhibition of p38 MAPK results in diminished pulmonary inflammation. *A*, Pulmonary histology of a saline-treated mouse. At 24 h following intratracheal instillation of saline, the murine lungs were fixed and stained with hematoxylin and eosin (see *Materials and Methods*). Plate represents a representative field from one of two mice at ×400 magnification. *B*, Histologic changes associated with intratracheal administration of LPS. Murine lung 24 h following intratracheal administration of LPS (300 ng/mouse). Plate depicts a representative field from one of four mice. *C*, Effect of in vivo p38 MAPK inhibition on LPS-induced histological changes. Mice were administered M39 by gastric intubation (see *Materials and Methods*) and then exposed to LPS in an identical manner as Fig. 4*B*. Plate depicts a representative field from one of four mice.

Characterization of murine pulmonary inflammation in response to intratracheal LPS

To study the role of p38 MAPK activation in the lungs, a model of mild pulmonary inflammation was developed. Following intratracheal administration of LPS, leukocytes and selected cytokines were quantified from BAL samples over a series of time points. A dose of LPS was selected that would elicit an exuberant neutrophil influx, followed by a secondary accumulation of mononuclear cells (primarily macrophages and monocytes), with near complete resolution by 72 h (Fig. 4*A*). The maximal neutrophil accumulation in the airspaces occurred at 24 h following LPS installation (Fig. 4*A*).

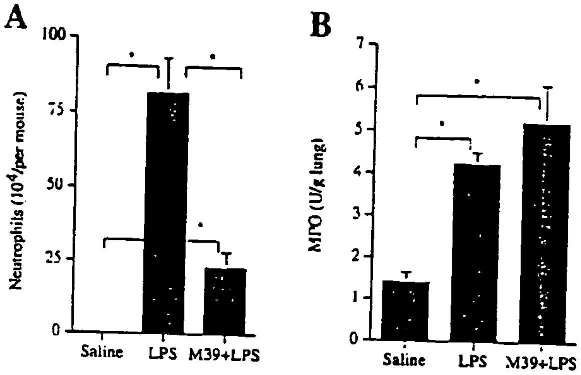

FIGURE 8. Systemic inhibition of p38 MAPK results in decreased accumulation of neutrophils in the airspaces independent of the whole lung. *A*, Neutrophil accumulation in airspaces. Neutrophils recovered by BAL at 24 h following intratracheal instillation of saline or LPS (300 ng/mouse) with and without administration of M39 by gastric intubation (identical with the conditions depicted in Fig. 7). Each bar represents mean number of neutrophils ± SEM from three animals. *, $p < 0.01$ by Student's t test comparing indicated condition. *B*, MPO content in lungs following BAL. To determine relative quantities of neutrophils present in the lungs excluding the neutrophils accumulated in the airspaces, the isolated lungs from the animals depicted in *A* were assayed for MPO following BAL. Each bar represents mean ± SEM of MPO activity per gram of lung tissue from three animals. *, $p < 0.01$ by Student's t test comparing indicated condition.

In association with administration of LPS, production of TNF-α, MIP-2, and KC peaked within 4 h, returning to baseline by 12 h (Fig. 4*B*). Cytokine recovery was negligible by 24 h, the point at which neutrophil influx was greatest, suggesting that cytokine release by neutrophils is minimal in this model.

Inhibition of p38 MAPK in vivo results in decreased leukocyte accumulation in the airspaces

To quantify changes in inflammation observed in the setting of in vivo p38 MAPK inhibition, we conducted BAL studies over 72 h following administration of LPS. Numbers of neutrophils and mononuclear cells recovered by BAL in mice following intratracheal LPS were counted. Administration of M39 resulted in significant reduction of neutrophil accumulation from 4 to 24 h (Fig. 5*A*). By 48 h, neutrophils were no longer present in the airways, but were replaced by monocytes/macrophages. When total mononuclear cells were evaluated, the effect of systemic p38 MAPK inhibition was not statistically significant (Fig. 5*B*). Together, these plots support the conclusion that in vivo inhibition of p38 MAPK result primarily in reduction of the early neutrophil accumulation, with little effect on the later recruitment of monocytes/macrophages.

Inhibition of p38 MAPK in vivo results in decreased TNF-α release in the airspaces

BAL studies of mice 0–72 h following administration of LPS were analyzed for TNF-α, MIP-2, and KC. Only TNF-α was found to be significantly reduced by in vivo inhibition of p38 MAPK with M39 (Fig. 6*A*), with no detectable change in the release of MIP-2 (Fig. 5*B*) or KC (Fig. 5*C*). This data suggests that systemic inhibition of p38 MAPK can have divergent effects on cytokine release, and that release of the KC and MIP-2 chemokines by resident pulmonary immune cells in the mouse is relatively less dependent on p38 MAPK signaling than TNF-α under the conditions studied.

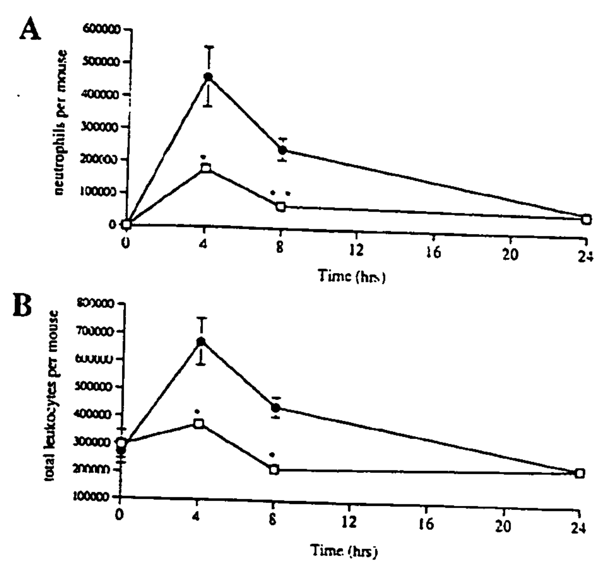

FIGURE 9. Effect of in vivo inhibition of p38 MAPK on KC-induced leukocyte accumulation and cytokine release. *A*, Neutrophil accumulation in the murine airspaces in response to intratracheal KC. Mice pretreated with M39 (□) were administered KC (1 μg) at time 0, and cell analysis of BAL was compared with untreated mice (●) over a series of times (see *Materials and Methods*). Each point represents mean number of neutrophils ± SEM from three to seven animals. Analysis of data by two-way ANOVA indicated a significant interaction between M39-induced inhibition and time, therefore the effect at each time point was analyzed separately. Significant inhibition (*, $p < 0.01$) was found at 4 h, with inhibition approaching significance at 8 h (**, $p = 0.028$). No significant changes were present at 24 h ($p = 0.95$). *B*, Leukocyte accumulation in the murine airspaces in response to KC. The total white blood cell count in BAL samples depicted in Fig. 8*A* was counted in mice administered M39 (□) compared with untreated mice (●) over a series of times. Each point represents mean number of cells ± SEM from three to seven animals. Analysis of data by two-way ANOVA indicated a significant interaction between M39-induced inhibition and time, therefore the effect at each time point was analyzed separately. Significant inhibition (*, $p < 0.01$) was found at 4 and 8 h. No significant changes were present at 24 h ($p = 0.99$).

Inhibition of p38 MAPK in vivo results in diminished histological evidence of pulmonary inflammation

The effect of in vivo p38 MAPK inhibition on the histological changes of mild LPS-induced pulmonary inflammation were evaluated. Animals were administered LPS intratracheally in the presence and absence of M39and compared with saline-treated controls. After 24 h, LPS-treated mice (Fig. 7*B*) demonstrated a significant interstitial and intraalveolar accumulation of leukocytes and edema when compared with saline treated animals (Fig. 7*A*). In the presence of 38 MAPK inhibition, inflammatory changes were evident, but to a lesser extent (Fig. 7*C*).

Inhibition of p38 MAPK selectively blocks the accumulation of neutrophils into the airspaces

Decreased neutrophil accumulation in the airspaces in response to LPS following inhibition of p38 MAPK could possibly be due to decreased retention of neutrophils in the pulmonary vasculature or lung interstitium, or by loss of the ability of the cells to migrate into the alveoli. The MPO assay was used to quantify the neutrophil burden in the pulmonary vasculature and interstitium. Animals were administered LPS intratracheally in the presence and absence of M39 and compared with saline-treated controls at 24 h, identical with the conditions depicted in Fig. 7, *A*–*C*. Neutrophil accumulation in the airspaces was determined by BAL (Fig. 8*A*), and

following BAL the isolated lungs were subjected to the MPO assay (Fig. 8*B*). Although significant reduction in neutrophil accumulation is observed in the setting of in vivo p38 MAPK inhibition (Fig. 8*A*), the amount of neutrophils present in the isolated lungs with or without p38 MAPK inhibition are equivalent (Fig. 8*B*). These results support the conclusion that systemic inhibition of p38 MAPK results in a loss of migration of neutrophils into the airways, consistent with the effects of in vitro p38 MAPK inhibition on neutrophil chemotaxis (Fig. 2).

Decreased neutrophil accumulation by inhibition of p38 MAPK in vivo occurs due to reduced neutrophil chemotaxic response

Observed decreases in pulmonary inflammation following systemic inhibition of p38 MAPK might occur due to diminished response of neutrophils to LPS, as a result of decreased TNF-α release (Fig. 6*A*) or as a selective inhibition of neutrophil chemotaxis (Fig. 2*B*). To evaluate the effect of systemic p38 MAPK inhibition on neutrophil chemotaxis independent of LPS and TNF-α, a model using KC-induced pulmonary inflammation was studied. KC is a potent and selective chemoattractant for murine neutrophils that triggers little of the inflammatory cascade. KC was administered intratracheally in mice in the presence and absence of M39, and BAL studies were performed from 0 to 48 h. Under the conditions studied, KC induced a rapid and self-limited migration of neutrophils that was significantly decreased by in vivo p38 MAPK inhibition (Fig. 9*A*). Unlike LPS, a substantial later accumulation of mononuclear cells did not occur in response to KC (Fig. 9*B*). BAL analysis demonstrated no measurable release of MIP-2 and TNF-α and levels of KC within the airspaces decreased rapidly following administration (data not shown). These results suggest that decreases in LPS-induced neutrophil accumulation following systemic inhibition of p38 MAPK can occur due to reduced neutrophil response, independent of TNF-α production.

Discussion

The murine model of lung inflammation described above uses a single, low-dose intratracheal administration of LPS to induce an acute inflammatory response characterized by rapid but self-limiting release of cytokines, followed by the transient influx of neutrophils and a secondary accumulation of mononuclear cells. This model was designed to study the critical early stages of lung inflammation in which neutrophil recruitment is a central feature. By limiting the extent of the initial insult, many of the features that contribute to the perpetuation of severe inflammation were avoided, including sustained release of cytokines, ongoing recruitment of leukocytes, significant damage of the parenchyma, and ultimately a significant mortality rate.

Through in vitro studies of various cell lines and primary cells, p38 MAPK has been linked to a variety of inflammatory responses. With the recent development of potent and specific inhibitors, the role of p38 MAPK in both in vitro systems and complex in vivo models of inflammation can be studied. Murine neutrophils were found to have nearly identical activation of p38α MAPK in response to stimulation by LPS with what has previously been reported in human neutrophils. Treatment of murine neutrophils with the novel p38 MAPK inhibitor M39 resulted in significant inhibition of p38α MAPK activity. Important functional effects of p38 MAPK inhibition in the murine neutrophil were the loss of chemotaxis toward MIP-2 and KC and the loss of TNF-α and MIP-2 release in response to LPS. Unexpectedly, parallel studies of murine AM demonstrated a 1000-fold greater concentration of M39 is required to block release of TNF-α, MIP-2, or KC. The greater sensitivity of neutrophils to inhibition of the p38 MAPK cascade was also observed in vivo. In response to intratracheal administration of LPS, the influx of neutrophils, but not mononuclear cells, was significantly decreased in the setting of systemic p38 MAPK inhibition. Quantification of the neutrophil accumulation in the whole lung demonstrated that under the conditions studied only the airspaces have a reduction of the influx of neutrophils, supporting the in vitro analysis of the dependence of neutrophil chemotaxis on p38 MAPK activation. Recovery of TNF-α in the airspaces was reduced through p38 MAPK inhibition, but quantities of MIP-2 and KC were not affected. When KC was used as a primary neutrophil chemoattractant, secondary release of TNF-α and MIP-2 was not evoked, but neutrophil influx was significantly blocked by systemic p38 MAPK inhibition.

Although the MAPK cascades are highly conserved, it is now understood that specific utilization of the MAPK cascades differs between neutrophils, macrophages, and other cells. In monocytes or macrophage cell lines, LPS has been reported to activate p42/44 (ERK) MAPK and JNK as well as the p38 MAPK cascade (31–33). Release of TNF-α by monocytes or macrophage cell lines can be blocked through selective inhibition of either the JNK (32), p38 MAPK (16), or the p42/44 (ERK) MAPK cascade (34). Disruption of a component of the p38 MAPK cascade in *MKK3*$^{-/-}$ mice failed to reduce TNF-α release by peritoneal macrophages in response to LPS (35). In T cells, inhibition of p38 MAPK has less of an effect on TNF-α release than does inhibition of the p42/44 (ERK) cascade (36). In contrast, LPS stimulation of neutrophils does not result in activation of the p42/44 (ERK) MAPKs or the JNKs (17, 18, 37). As a short-lived, terminally differentiated primary cell, the neutrophil possesses a more limited synthetic capability and, in response to LPS, uses fewer of the available intracellular signal transduction mechanisms. Thus, the selective loss of neutrophil function in the setting of systemic p38 MAPK inhibition suggests that neutrophils are relatively more dependent on signal transduction via the p38 MAPK cascade than AM.

Although considerable recent interest has been focused on the activation and function of p38 MAPK, there are few reports of in vivo inhibition of this signaling pathway. The pyridinyl imadazole compounds, including SB203580 and SK&F86002, are the most widely studied p38 MAPK inhibitors and have been shown to possess antiinflammatory properties in animal models. These early p38 MAPK inhibitors were shown to reduce neutrophil influx in response to monosodium urate- or carrageenan-induced peritonitis (38) and collagen-induced arthritis (39, 40). In response to i.p. injection of LPS, administration of these compounds resulted in decreased recovery of TNF-α by peritoneal washout (41) and decreased serum TNF-α and mortality in a murine model of endotoxin shock (40, 42, 43). The antiinflammatory effects of these p38 MAPK inhibitors occurred in the absence of generalized immunosupression (40, 41, 44, 45). The effects of systemic p38 MAPK inhibition on pulmonary inflammation has not been described. To date, nearly all studies of the functional role of p38 MAPK have used the compound SB203580, which has an $IC_{50} = 39 \pm 11$ nM for p38 MAPK as well as considerable inhibitory effects toward c-Raf ($IC_{50} = 330 \pm 155$ nM) and JNK2α1 ($IC_{50} = 290 \pm 110$ nM). In comparison, M39 has an $IC_{50} = 0.11 \pm 0.046$ nM for p38 MAPK and is significantly less active toward c-Raf ($IC_{50} = >1000$ nM) or JNK2α1 ($IC_{50} = 675$ nM) (25). As a more potent and selective p38 MAPK inhibitor, M39 is better suited for in vivo studies than previously available compounds.

In the murine model of mild LPS-induced pulmonary inflammation, the predominant effect of in vivo p38 MAPK inhibition was to reduce recruitment of neutrophils. Based on in vitro chemotaxis assays to MIP-2 and KC, it would appear that this effect

can occur as a result of decreased neutrophil response. BAL measurement of chemokine levels support this conclusion, as neither KC nor MIP-2 release was decreased in animals treated with M39. In addition, in vivo inhibition of p38 MAPK blocked neutrophil accumulation in response to intratracheal administration of KC, independent of LPS or TNF-α. Although decreased TNF-α release following LPS stimulation was detected in animals treated with M39, this effect appears to be of secondary importance under the conditions studied. The apparently greater dependence of neutrophils on p38 MAPK signaling when compared with resident cells of the lung suggests the potential for selective analysis and modulation of neutrophil influx in pulmonary inflammation.

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